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Effect of bisphosphonates on vascular calcification and bone metabolism in experimental renal failure

Koba A. Lomashvili¹, Marie-Claude Monier-Faugere², Xiaonan Wang¹, Hartmut H. Malluche² and W. Charles O'Neill¹

¹Renal Division, Emory University School of Medicine, Atlanta, Georgia, USA and ²Division of Nephrology, Bone and Mineral Metabolism, University of Kentucky, Lexington, Kentucky, USA

Although it is known that bisphosphonates prevent medial vascular calcification *in vivo*, their mechanism of action remains unknown and, in particular, whether they act directly on the blood vessels or indirectly through inhibition of bone resorption. To determine this, we studied the effects of two bisphosphonates on calcification of rat aortas *in vitro* and on *in vivo* aortic calcification and bone metabolism in rats with renal failure. We produced vascular calcification in rats with adenine-induced renal failure fed a high-phosphate diet. Daily treatment with either etidronate or pamidronate prevented aortic calcification, with the latter being 100-fold more potent. Both aortic calcification and bone formation were reduced in parallel; however, bone resorption was not significantly affected. In all uremic rats, aortic calcium content correlated with bone formation but not with bone resorption. Bisphosphonates also inhibited calcification of rat aortas in culture and arrested further calcification of precalcified vessels but did not reverse their calcification. Expression of osteogenic factors or calcification inhibitors was not altered by etidronate *in vitro*. Hence, these studies show that bisphosphonates can directly inhibit uremic vascular calcification independent of bone resorption. The correlation between inhibition of aortic calcification and bone mineralization is consistent with a common mechanism such as the prevention of hydroxyapatite formation and suggests that bisphosphonates may not be able to prevent vascular calcification without inhibiting bone formation in uremic rats.

Kidney International (2009) **75**, 617–625; doi:10.1038/ki.2008.646; published online 7 January 2009

KEYWORDS: bone formation; bone histomorphometry; bone resorption; pyrophosphate; rat aorta; uremic osteodystrophy

Calcification of the medial layer of arteries is common in renal failure and is thought to contribute to the increased incidence of cardiovascular disease in this population. Recent studies have shown that medial calcification is a far more complex process than simple precipitation of calcium phosphate. It is clear that several endogenous inhibitors of hydroxyapatite formation are responsible for preventing vascular calcification under normal conditions¹ and must be deficient in pathologic calcification. Vascular calcification is also associated with osteogenic changes in smooth muscle² and with altered bone metabolism,³ suggesting additional pathophysiologic mechanisms. We have shown that pyrophosphate (PPi), a potent inhibitor of hydroxyapatite formation,^{4–6} is produced by smooth muscle in quantities sufficient to prevent medial calcification *in vitro*.⁷ Plasma PPi levels are reduced in hemodialysis patients⁸ and PPi levels in the vascular wall may be reduced in uremia by virtue of increased alkaline phosphatase activity.⁹ Humans and mice with low levels of PPi due to the absence of PC-1, a membrane-bound ecto-nucleotide pyrophosphatase/phosphodiesterase that produces PPi,^{10–12} develop severe, fatal arterial calcification, indicating that PPi deficiency could contribute to vascular calcification in renal failure.

Systemic administration of PPi to vitamin D-toxic rats prevents vascular calcification¹³ but this approach is limited by the susceptibility of PPi to hydrolysis. Bisphosphonates are nonhydrolyzable analogs of pyrophosphate that were originally developed to treat ectopic calcification. Although early compounds were successful at preventing vascular calcification in rats,^{6,14,15} inhibition of bone formation limited this approach in humans.¹⁵ It was assumed that the prevention of vascular calcification as well as the inhibition of bone formation were due to a physicochemical effect of the bisphosphonates related to their PPi-like structure. Subsequent chemical modifications imparted an additional biological action that yielded newer bisphosphonates that are far more potent at inhibiting bone resorption than bone formation. Recent studies with these compounds have shown that medial vascular calcification produced by vitamin D, warfarin, or renal failure in rats can be prevented at relative potencies that match those for inhibition of bone resorption rather than bone formation.^{16–18}

Correspondence: W. Charles O'Neill, Renal Division, Emory University, WMB 338, 1639 Pierce Dr., Atlanta, Georgia 30322, USA. E-mail: woneill@emory.edu

Received 14 January 2008; revised 15 October 2008; accepted 21 October 2008; published online 7 January 2009

Based on these results, it has been assumed that the prevention of vascular calcification is related to the antiresorptive activity of bisphosphonates in bone, thus supporting the putative link between bone resorption and vascular calcification. However, effects on bone were not examined in these studies and bisphosphonates can inhibit calcium deposition in vascular smooth muscle cells in culture.¹⁹ Thus, the possibility that bisphosphonates directly inhibit vascular calcification in renal failure independent of effects on bone cannot be excluded. Whether calcification can be prevented without reducing bone mineralization is also unknown. Using two bisphosphonates with different relative potencies for inhibition of bone resorption and formation, we compared vascular calcification and bone metabolism *in vivo* in rats with renal failure. An *in vitro* model of vascular calcification in cultured rat aortas was used to examine direct effects of bisphosphonates independent of bone.

RESULTS

Rats were fed with adenine for 28 days together with a high-phosphate diet to induce renal failure and vascular calcification. One rat treated with the highest dose of etidronate was killed on day 22 due to distress. One rat receiving the lowest dose of pamidronate died on day 27 and was not included in the results. The resulting plasma concentrations of urea, calcium, and phosphate in rats receiving etidronate, pamidronate, or vehicle are shown in Figure 1. Plasma urea increased 2.5- to 4-fold in vehicle-treated, adenine-fed rats compared with pair-fed control rats. Pamidronate did not alter the plasma urea concentration at any dose but it was significantly increased at the two highest etidronate doses. Plasma urea did not differ between rats treated with etidronate or pamidronate at similar doses. The plasma phosphate concentration was substantially elevated in uremic rats and was slightly decreased and substantially increased at the highest and lowest doses of etidronate respectively. Plasma phosphate was not significantly altered by pamidronate although it was lower than in etidronate-treated rats. Plasma calcium was not altered by uremia or pamidronate but was significantly reduced at the highest etidronate dose. At similar doses, the plasma calcium concentration was lower in the pamidronate-treated rats. Plasma parathyroid hormone concentration was markedly elevated in uremic rats but not altered by bisphosphonates.

Extensive aortic calcification developed in half of the uremic rats treated with vehicle alone. As shown in Figure 2, both bisphosphonates reduced aortic calcification. Complete inhibition was obtained at 0.08 $\mu\text{mol/kg}$ per day of pamidronate and at 8 $\mu\text{mol/kg}$ per day of etidronate. Samples of bone were examined from the same rats. Bone volume, osteoid volume, and cortical thickness were not altered in uremic rats and were unaffected by bisphosphonate (Table 1). Parameters of bone resorption are shown in Figure 3. Erosion depth and osteoclast number both increased (but not significantly) in adenine-fed rats compared with pair-fed nonuremic rats and tended to be higher in etidronate-treated

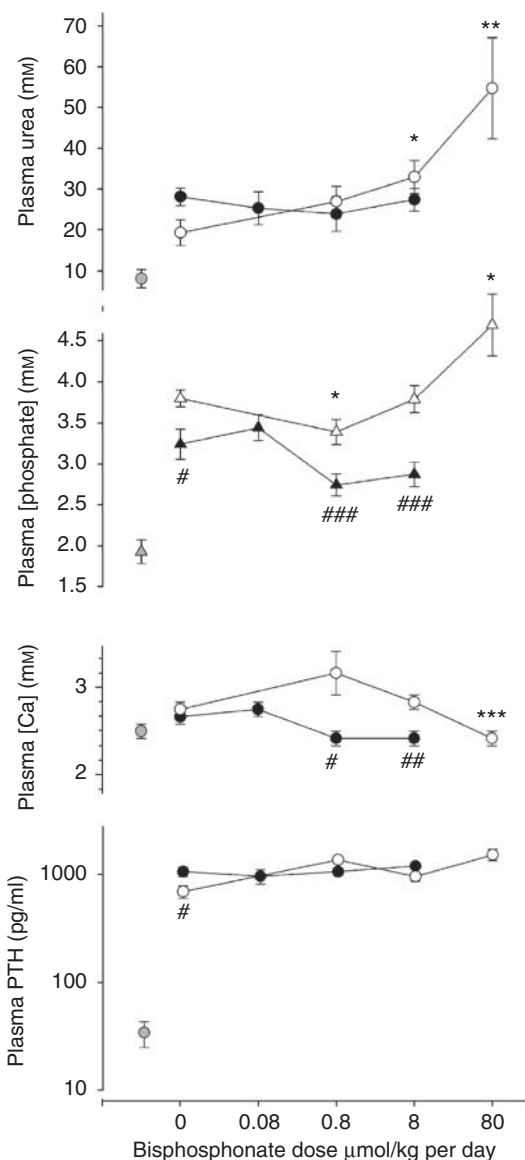


Figure 1 | Plasma chemistries in bisphosphonate-treated rats.

Open symbols, etidronate-treated rats; solid symbols, pamidronate-treated rats; shaded symbols: nonuremic rats. There are separate untreated uremic controls for pamidronate and etidronate-treated rats. * $P < 0.05$, ** $P < 0.02$, *** $P < 0.005$ versus untreated adenine-fed rats; # $P < 0.05$, ## $P < 0.02$, ### $P < 0.01$ versus etidronate-treated rats.

rats than in pamidronate-treated rats. Although there was a decreasing trend with increasing doses of bisphosphonate, none of the changes were significant. Parameters of bone formation are presented in Figure 4. Both bone formation rate (2.7-fold; $P = 0.0009$) and osteoblast number (20-fold; $P = 0.0013$) were increased in the adenine-fed rats. Bisphosphonates completely eliminated bone formation, with pamidronate being at least 10-fold more potent, but osteoblast number was reduced only at the highest dose of etidronate. Mineralizing surface (1.5-fold; $P = 0.05$) was also increased in the adenine-fed rats and substantially reduced with bisphosphonates, again with pamidronate being at least 10-fold more potent (Table 1).

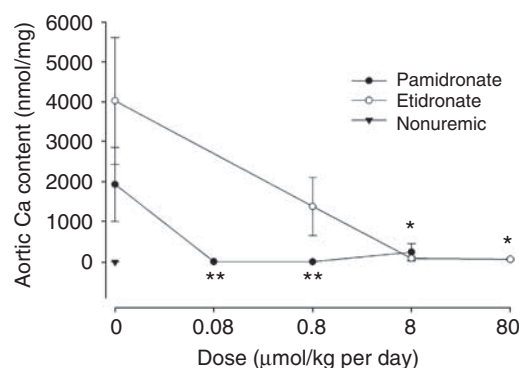


Figure 2 | Effect of bisphosphonates on aortic calcification in renal failure. Solid symbols, pamidronate-treated rats; ** $P < 0.05$ versus untreated uremic rats by Mann-Whitney U -test. Open symbols, etidronate-treated rats; * $P < 0.02$ versus untreated uremic rats by Mann-Whitney U -test.

Table 1 | Bone parameters in control and bisphosphonate-treated uremic rats

Treatment	Dose μmol/kg per day	Bone volume %	Cortical thickness μm	Osteoid thickness μm	Mineralization surface %
No adenine		12.5 ± 1.6	494 ± 34	3.64 ± 0.50	13.0 ± 1.4
Adenine+ etidronate	0	14.5 ± 2.1	594 ± 31	3.82 ± 0.58	21.4 ± 3.8
	0.8	10.9 ± 1.1	637 ± 28	5.04 ± 1.0	21.7 ± 2.6
	8	14.6 ± 1.1	649 ± 20	12.2 ± 3.0**	9.2 ± 1.5**
	80	15.5 ± 1.3	621 ± 27	6.43 ± 1.5	3.3 ± 0.9***
Adenine+ pamidronate	0	13.8 ± 2.3	478 ± 31	4.36 ± 0.45	19.7 ± 2.8
	0.08	10.4 ± 0.6	540 ± 29	6.23 ± 2.2	10.3 ± 1.6**
	0.8	10.3 ± 1.7	525 ± 23	3.27 ± 0.61	9.6 ± 2.1*
	8	13.9 ± 2.1	466 ± 18	5.75 ± 1.7	1.4 ± 0.2***

* $P < 0.05$, ** $P < 0.02$, *** $P < 0.005$ vs untreated uremic.

Parameters of bone formation and resorption were compared with aortic calcium content by linear regression in the entire cohort of adenine-fed rats (Figure 5). Owing to the large range of values for aortic calcium, regression was performed using the logarithm of calcium content to minimize skewing of the data. Of all the parameters of bone formation examined, bone formation rate correlated best with aortic calcium content (Figure 5, top). The correlation between aortic calcium content and bone formation rate was 0.41 (0.48 when the outlying value was omitted). Aortic calcium also correlated significantly with osteoblast surface ($r = 0.31$), osteoblast number ($r = 0.35$), and mineralizing surface ($r = 0.34$). In contrast, there was no significant correlation between aortic calcium content and osteoclast number (Figure 5, bottom). Correlation with other parameters of bone resorption was even poorer (not shown).

To determine whether bisphosphonates can affect vascular calcification directly, independent of effects on bone, studies were repeated in normal rat aortas in culture. Culture of rat aortas with alkaline phosphatase results in medial calcification after 9 days by virtue of removing pyrophosphate.⁷

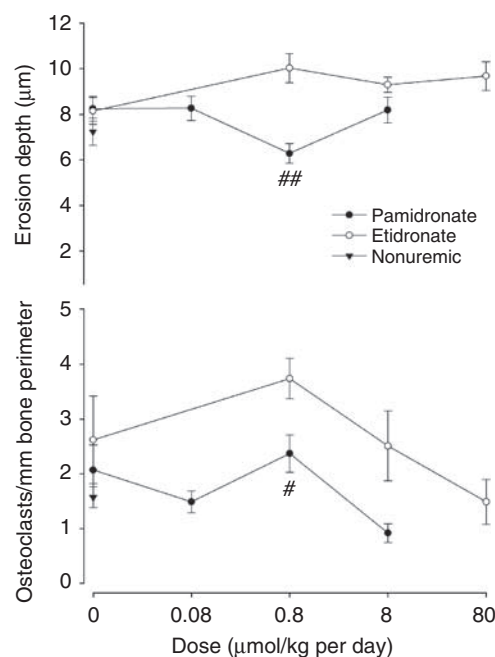


Figure 3 | Parameters of bone resorption in bisphosphonate-treated rats. Top: erosion depth. Bottom: osteoclast number. Solid symbols, pamidronate-treated; open symbols, etidronate-treated rats; triangles: control, nonuremic rats. There are separate untreated uremic controls for pamidronate and etidronate-treated rats. # $P < 0.02$, ## $P < 0.005$ versus etidronate-treated rats.

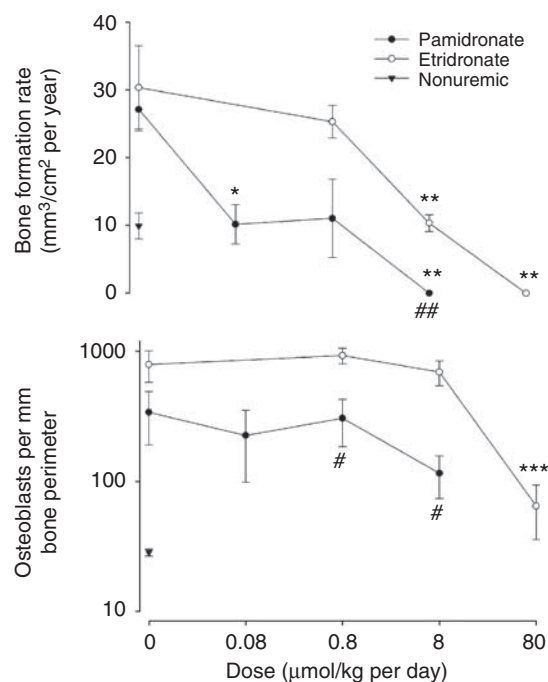


Figure 4 | Parameters of bone formation in bisphosphonate-treated rats. Top: Bone formation rate per bone surface. Bottom: Number of osteoblasts per mm bone perimeter. There are separate untreated uremic controls for pamidronate and etidronate-treated rats. * $P < 0.01$, ** $P < 0.005$, *** $P < 0.002$ versus, control uremic rats; # $P < 0.01$, ## $P < 0.005$ versus etidronate-treated rats.

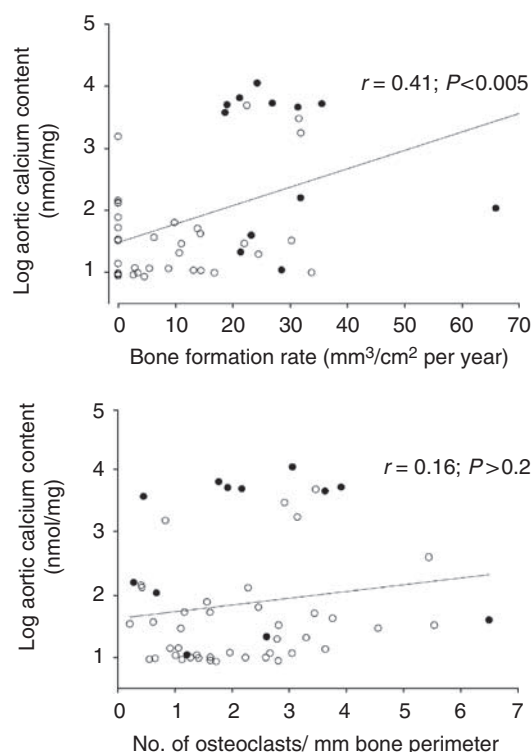


Figure 5 | Correlation between aortic calcium content and bone histomorphometry in uremic rats treated with or without bisphosphonates. Top: bone formation rate per bone surface; bottom: number of osteoclasts per bone perimeter. Open symbols: rats treated with etidronate or pamidronate. Solid symbols: rats treated with vehicle alone. Lines are linear regressions.

Warfarin, which increases calcification *in vitro* by reducing levels of matrix Gla protein (KA Lomashvili and WC O'Neill, unpublished data), was added to maximize calcification and provide a more precise comparison between the two agents. As shown in Figure 6a, calcification was prevented by increasing concentrations of etidronate or pamidronate, with complete inhibition at 0.1 and 1 μM , respectively. Inhibition of calcification was also observed in the absence of MGP (not shown). Medial calcification of aortas can also be produced in culture without alkaline phosphatase when the vessels are injured prior to culture by rubbing with a cotton swab. As shown in Figure 6b, substantially greater concentrations of pamidronate were required to inhibit calcification in injured aortas than in uninjured aortas cultured with alkaline phosphatase. A similar decrease in potency in injured aortas was observed with etidronate (not shown).

As bisphosphonates have been reported to inhibit alkaline phosphatase,²⁰ reduction of PPi hydrolysis by alkaline phosphatase could be a potential mechanism for the prevention of calcification by bisphosphonates. However, hydrolysis of PPi by rat aortas was not altered by 10 μM pamidronate (data not shown), a concentration that completely prevented calcification in cultured aortas. Studies were also performed to determine whether bisphosphonates

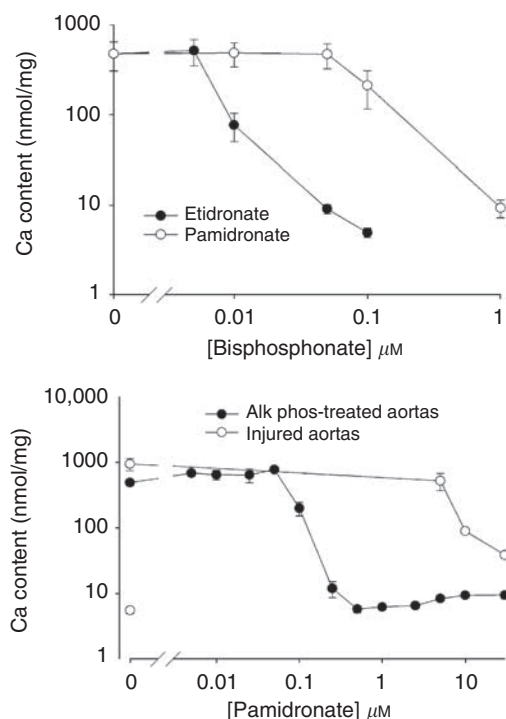


Figure 6 | Effect of bisphosphonates on calcification of rat aortas in culture. Top: Aortas were incubated in DMEM containing 3.8 mM phosphate, 3.75 U/ml of alkaline phosphatase, 25 μM warfarin, and the indicated concentrations of bisphosphonate for 9 days. Results are the means \pm s.e. of seven aortic rings. Bottom: Injured and uninjured aortas were incubated in DMEM with 3.8 mM phosphate, with or without 3.75 U/ml of alkaline phosphatase, and the indicated concentrations of pamidronate for 9 days. Results are the means \pm s.e. of at least three aortic rings.

Table 2 | Abundance of mRNA in aortas cultured with or without 1 μM etidronate

	3 days (low phosphate without AP)			9 days (high phosphate with AP)		
	Etidronate	Control	P	Etidronate	Control	P
MGP	776 \pm 66	888 \pm 76	NS	1760 \pm 729	2630 \pm 1110	NS
OPN	194 \pm 26	128 \pm 21	NS	1420 \pm 494	1770 \pm 634	NS
Runx2	1.7 \pm 0.4	1.6 \pm 0.5	NS	17 \pm 3	68 \pm 40	NS
Osterix	0.07 \pm 0.03	0.038 \pm 0.024	NS	<0.01	<0.01	NS

AP, alkaline phosphatase; MGP, matrix gla protein; OPN, osteopontin. Values are copies per 10⁵ copies of 18S RNA.

alter the expression of calcification inhibitors or osteogenic factors in vascular smooth muscle (Table 2). Messenger RNA was measured by quantitative polymerase chain reaction and normalized to 18S RNA. Substantial quantities of mRNA for both matrix Gla protein and osteopontin were detected after 3 days in normal medium and even higher levels were observed after 9 days in high-phosphate medium with alkaline phosphatase (calcifying conditions). The levels in aortas cultured for 9 days in normal medium were similar to

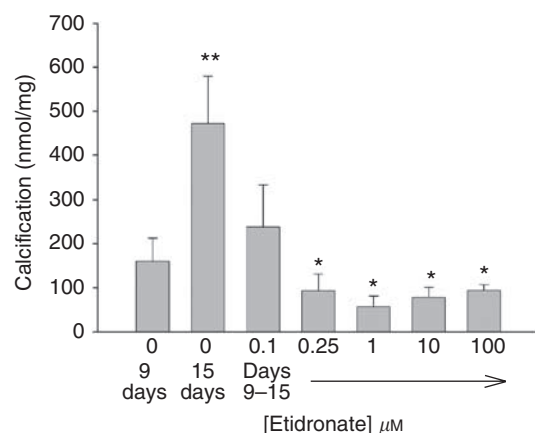


Figure 7 | Arrest of aortic calcification by etidronate. Aortas were cultured with alkaline phosphatase and ^{45}Ca for 9 days and then for an additional 6 days with alkaline phosphatase in the presence of varying concentrations of etidronate. Calcium content of the aortas was measured as described in 'Materials and Methods'. Results are the means \pm s.e. of at least eight aortic rings. * $P < 0.001$ versus 15 days without etidronate; ** $P < 0.02$ versus 9 days.

those after 3 days (not shown). In contrast, expression of the transcription factor *runx2* (*cbfa-1*) was very low after 3 days in normal medium, consistent with levels in fresh aorta (not shown). The slightly higher levels in aortas cultured for 9 days under calcifying conditions were also observed in aortas cultured for 9 days in normal medium (not shown). Expression of the bone-specific transcription factor *osterix* was extremely low. Etidronate at $1\text{ }\mu\text{M}$ did not alter the content of any of the mRNAs at either 3 days in normal medium or 9 days under calcifying conditions.

Additional studies were performed to determine whether bisphosphonates could also arrest or reverse existing calcification. To examine the effect on progression of calcification, aortas were cultured with alkaline phosphatase for 9 days in the absence of etidronate and then cultured an additional 6 days with alkaline phosphatase and varying concentrations of etidronate (Figure 7). Culture for an additional 6 days without etidronate resulted in a further 195% increase in calcification ($P < 0.02$). This increase was prevented by etidronate but at higher concentrations (approximately 10-fold) than required to prevent *de novo* calcification. There was no further reduction at etidronate concentrations above $0.25\text{ }\mu\text{M}$. Although calcification at 15 days was less than the calcification at 9 days at maximum concentrations of etidronate, suggesting reversal, the difference was not significant. To examine reversibility of calcification directly, release of ^{45}Ca was measured in precalcified aortas. The results are shown in Figure 8, with the fraction of ^{45}Ca remaining fitting a biexponential decline ($r > 0.99$). Approximately 14% of the calcium exhibited a half-life of $16.0 \pm 0.8\text{ h}$ while the bulk of the calcium had a much longer half-life of $2920 \pm 351\text{ h}$. Neither half-life was altered by $30\text{ }\mu\text{M}$ etidronate ($14.0 \pm 0.8\text{ h}$ and $2980 \pm 421\text{ h}$).

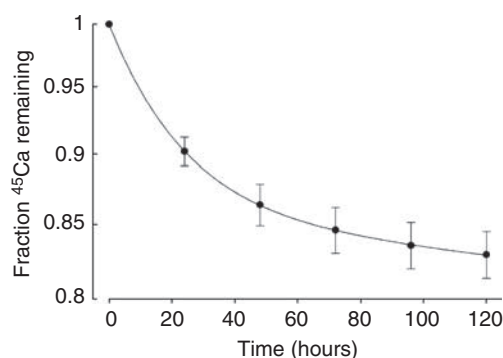


Figure 8 | Washout of calcium from calcified vessels. Injured aortas were calcified for 9 days in high-phosphate DMEM prior to washout as described in 'Materials and Methods'. Results are expressed as the fraction of ^{45}Ca remaining in the aortas and are the means \pm s.e. of determinations in eight aortic rings.

DISCUSSION

Both etidronate and pamidronate prevented aortic calcification in uremic rats, confirming previous studies of bisphosphonates in the same model¹⁸ and other models^{6,16,17} of vascular calcification. Inhibition of calcification cannot be explained by changes in the circulating phosphate or calcium levels. Etidronate completely prevented calcification at $8\text{ }\mu\text{mol/kg}$ per day despite plasma calcium and phosphate levels that were almost identical to levels in untreated rats. Although the plasma calcium concentration was decreased at the highest dose of etidronate, any effect on calcification would have been offset by the concurrent increase in phosphate concentration. Plasma calcium and phosphate levels were reduced at the two highest doses of pamidronate compared with untreated rats, but the differences were not significant. Furthermore, pamidronate completely suppressed calcification at the lowest dose despite no change in plasma calcium or phosphate concentration. Although a decrease in plasma calcium might have been expected based on clinical experience, the bisphosphonates had little effect on bone resorption and the inhibition of bone formation would have countered any decrease in circulating calcium levels. Bisphosphonates also failed to lower calcium levels in a previous study in uremic rats.²¹ The higher phosphate and lower calcium levels at the highest etidronate dose probably reflect worse renal failure as indicated by the higher urea level. This is consistent with reports of renal failure with high doses of bisphosphonates.²²

The fact that the potency of different bisphosphonates in previous studies of vascular calcification paralleled their reported antiresorptive potencies *in vivo* has been taken as evidence that medial vascular calcification is linked to bone resorption. However, no bone histology has been presented to support this hypothesis. The relative potencies of etidronate and pamidronate in the current study also paralleled the reported potencies for inhibition of bone resorption, but bone histology did not support a link between inhibition of vascular calcification and inhibition

of bone resorption. The effect of bisphosphonate on indices of bone resorption was small and is consistent with a previous study in rats with renal ablation.²¹ Doses of bisphosphonates that completely suppressed aortic calcification did not significantly reduce any index of bone resorption. Furthermore, there was no consistent correlation between aortic calcium content and any index of bone resorption in the entire cohort of uremic rats.

The small increases in parameters of bone resorption compared with the larger increases in bone formation rate in the untreated uremic rats do not necessarily indicate a net increase in bone in this model. Bone resorption cannot be directly measured and an increase in bone volume was not observed histologically. Furthermore, this model is superimposed on a skeleton that continues to increase during the life of the rats. This together with the short duration of the model may explain why bone volume did not decrease with uremia.

The mechanism by which bisphosphonates reduce bone resorption and why the effect varies so widely among different compounds is not completely understood. The fact that the different potencies *in vivo* are not always reproduced *in vitro*¹⁵ suggests that differences in pharmacokinetics may contribute. Bioavailability can vary due to differences in binding to proteins, soft tissues, and bone^{15,23} and the pharmacokinetics of subcutaneous administration as used in rats have not been examined. Although the inhibition of bone resorption has been documented in several conditions associated with increased bone turnover, there has been only one previous study of bisphosphonate therapy in uremic osteodystrophy.²¹ Weekly treatment with ibandronate in partially nephrectomized rats did not alter osteoclast numbers or erosion surface but did reduce erosion depth. However, as in the present study, there were greater reductions in bone formation. As formation and resorption are normally coupled, the decrease in bone formation could represent a restoration of normal bone turnover. This is unlikely since, in this study, formation was reduced to levels well below control (nonuremic) animals. The results of these two studies suggest that caution must be used in extrapolating reported potencies of bisphosphonates for bone resorption and bone formation to bone metabolism in renal failure. As rat bone lacks the haversian system present in humans, the results cannot necessarily be extrapolated to patients with renal failure.

Inhibition of aortic calcification actually correlated better with inhibition of bone formation, which was almost completely suppressed by bisphosphonate. The potency of different bisphosphonates in inhibiting bone formation varies much less than the potency for inhibiting bone resorption,^{15,23} owing to the fact that inhibition of mineralization is a direct effect on hydroxyapatite formation related to the pyrophosphate-like backbone¹⁵ and not influenced as much by the side groups.²³ The results in uremic rats showing decreased bone formation and mineralizing surface without a decrease in osteoblast number is consistent with this

mineralizing defect. The fact that osteoid thickness did not increase is probably due to the short duration of treatment. The greater potency of pamidronate compared to etidronate *in vivo* may relate to pharmacokinetics since it is less potent than etidronate on bone *in vitro*²³ and was less effective at inhibiting calcification of aortas *in vitro* in the current study. The frequent (daily) dosing combined with decreased clearance due to renal failure may also have contributed to the potency of pamidronate *in vivo* as continuous presence of pamidronate versus intermittent exposure greatly increases inhibition of bone mineralization *in vitro*.²³ The correlation between aortic calcification and bone formation suggests that the two are linked, but it is also possible that inhibition of vascular calcification is a direct effect of bisphosphonates on hydroxyapatite formation that also occurs in the bone.

The fact that both bisphosphonates also prevented calcification of rat aortas in culture clearly demonstrates direct inhibition independent of the bone. Inhibition of the aortic calcification induced by alkaline phosphatase *in vitro* is consistent with the fact that bisphosphonates can substitute for the pyrophosphate removed by alkaline phosphatase and are resistant to the action of this enzyme. Bisphosphonates also inhibited calcification of injured aortas cultured in the absence of alkaline phosphatase, although higher concentrations were required. The mechanism of the calcification in injured aortas is not clear but upregulation of alkaline phosphatase does occur.⁷ Calcification is usually more robust in injured aortas, which may require greater quantities of bisphosphonates as inhibition likely requires binding to the hydroxyapatite.²⁴ The greater potency of etidronate compared with pamidronate *in vitro* is consistent with results in the bone²³ and indicates a direct physicochemical action of bisphosphonates on hydroxyapatite formation. Pamidronate contains a side group on the carbon atom that imbues it with an additional, intracellular action that is thought to be responsible for the greater inhibition of bone resorption.¹⁵ This clearly does not enhance inhibition of aortic calcification *in vitro* and appears to interfere with the physicochemical action.

In addition to preventing *de novo* calcification *in vitro*, bisphosphonates were also effective in arresting progression of established calcification. This required significantly greater amounts, which may be related to the extent of calcification. Bisphosphonates bind avidly to hydroxyapatite²⁴ and this is probably the mechanism by which calcification is blocked. Thus the amount required to prevent calcification is probably dependent on the amount of hydroxyapatite present, which has been demonstrated *in vitro* with PPI.⁵ Although etidronate blocked further calcification, it did not directly reverse calcification. The kinetics of calcium release from calcified vessels revealed two pools. The majority of the calcium was released very slowly with a half-life of 40–120 days. This undoubtedly represents apatitic calcium, which is highly insoluble under physiologic conditions.²⁵ The remainder of the calcium was released more rapidly and probably represents amorphous calcium phosphate or

brushite, which is far more soluble.²⁶ Although etidronate did not increase the rate of either pool, reversal of calcification could occur over an extended period of time as the calcifications dissolve when further hydroxyapatite formation is prevented. The lack of effect on dissolution is in contrast to the previously reported inhibition of hydroxyapatite dissolution by bisphosphonates at similar concentrations.²⁷ Possible explanations for this difference are that the previous study used chemically prepared hydroxyapatite, was not performed in a biological system, and was performed in the absence of calcium or phosphate. Physiologic concentrations of calcium and phosphate were used in the present studies to mimic conditions *in vivo* and to maintain the health of the cells. Thus, some or all of the ⁴⁵Ca release could represent exchange rather than net release of calcium.

Alkaline phosphatase has a critical role in calcification by virtue of hydrolyzing PPi.^{7,28} Although bisphosphonates are not hydrolyzable, they could interact with the catalytic site and interfere with PPi hydrolysis, resulting in higher PPi levels and inhibition of calcification. Such inhibition has been reported but required millimolar concentrations.²⁰ In the current studies, pamidronate did not alter the rate of hydrolysis of PPi by aortas, indicating that this is not a mechanism for preventing calcification. Another key protein is matrix Gla protein (MGP), an inhibitor of vascular calcification.²⁹ Despite a report that bisphosphonates reduce expression of mRNA for MGP in cultured smooth muscle cells,¹⁹ no changes were detected in cultured aortas. There was also no change in the amount of mRNA for osteopontin, another inhibitor.³⁰ The increased abundance of these mRNAs during culture under calcifying conditions is consistent with the increased protein expression that is observed (Lomashvili KA and O'Neill WC, unpublished studies). Etidronate also did not alter the content of mRNA for runx2, an osteogenic transcription factor that has been hypothesized to drive smooth muscle calcification,³¹ or osterix, a bone-specific transcription factor. Of note, the abundance of both mRNAs was very low in aortas and did not increase under calcifying conditions. These negative data all point to direct inhibition of hydroxyapatite formation as the mechanism for bisphosphonate action in cultured aortas.

This study demonstrates that inhibition of uremic vascular calcification by bisphosphonates correlates more strongly with inhibition of bone formation than inhibition of bone resorption. Although it is possible that there is a direct link between bone formation and vascular calcification, the *in vitro* data indicate that bisphosphonates directly inhibit hydroxyapatite formation at both locations. This suggests that doses of bisphosphonates necessary to prevent vascular calcification will also impair bone formation, thus limiting their therapeutic potential. However, aortic calcification was prevented at a dose of pamidronate that did not suppress bone formation below normal levels, indicating that a therapeutic window may exist. The lowest dose of pamidronate used in this study (0.03 mg/kg per day for 28 days) is similar to the dose in humans to inhibit bone resorption

(90 mg monthly). However, subcutaneous administration may not be equivalent to intravenous injection in humans and pamidronate clearance may be significantly retarded by renal failure. Also, this model of renal failure and vascular calcification in rats may not be applicable to humans with renal failure because of the nature of the renal lesion, the short duration of uremia, and the differences between rat and human bone. Although it is difficult to compare these doses, it is likely that the doses, which inhibit vascular calcification are substantially higher than the antiresorptive doses currently used in humans. Additional studies with careful attention to drug bioavailability, dynamics of vascular calcification, and changes in bone metabolism will be required to determine whether bisphosphonates are clinically useful in preventing uremic vascular calcification. Although this study does not provide evidence for a causal relationship between bone metabolism and vascular calcification, it does not refute this putative link. As bisphosphonates have direct effects on vascular calcification, they cannot be used to address this hypothesis.

MATERIALS AND METHODS

Animal studies

Chronic renal failure was produced by feeding adenine to rats.^{32,33} This results in crystallization of 2,8-dihydroxyadenine in the tubules and interstitium with resulting interstitial nephritis and fibrosis and all the metabolic abnormalities of chronic renal failure.³² Male Sprague-Dawley rats were obtained from Charles River Laboratories and fed a standard rodent diet until body weight exceeded 300 g. Rats were then fed a 2.5% protein diet (Harlan Teklad, Madison, WI, USA), which enhances vascular calcification,¹⁸ supplemented with phosphate. This diet contained 1.06% calcium and 0.92% phosphorus. The adenine content was 0.75% for the first 7 days and 0.5% thereafter. Control rats were fed the identical diet without adenine but restricted in amount so that their weight loss matched that of the adenine-fed rats. Etidronate (Sigma-Aldrich, St Louis, MO, USA) and pamidronate (Novartis, Basel, Switzerland) were administered in saline, buffered to pH 7.4, by daily subcutaneous injection starting on the first day of the adenine diet. The doses of etidronate were 0.17, 1.7, and 17 mg/kg per day (0.8, 8, and 80 μ mol/kg per day) and the doses of pamidronate were 0.03, 0.3, and 3 mg/kg per day (0.08, 0.8, and 8 μ mol/kg per day). There were seven rats in each group with renal failure and six pair-fed rats without renal failure. Rats were killed after 28 days and aortas were perfused with saline, harvested, and cleaned. After drying and weighing, the aortas were extracted overnight in 1 M HCl and calcium in the extract was measured colorimetrically by the cresolphthalein method.³⁴ Plasma urea was measured colorimetrically by the urease-glutamate dehydrogenase method (Sigma-Aldrich, St Louis, MO), plasma phosphate was measured colorimetrically by the molybdate method,³⁵ and plasma calcium was measured by the cresolphthalein method.³⁴ Plasma intact parathyroid hormone levels were determined using a rat enzyme-linked immunoassay (Scantibodies Inc., Santee, CA, USA).

Bone histomorphometry

Rats received intraperitoneal injections of calcein (2 mg) on days 20 and 25. At the time of killing, one femur was removed, cleaned of attached tissue, and placed in absolute ethanol. After dehydration,

samples were embedded in methyl methacrylate and eight sections were cut serially at 4 µm thickness with a heavy-duty microtome. This thickness allows analysis of the same histologic features in slides prepared for light and fluorescent microscopy. Every other section was stained with the modified Masson–Goldner trichrome technique whereas the other sections were reserved for fluorescence microscopy. Static and dynamic parameters of bone structure, formation, and resorption were measured at a standardized site below the growth plate using a semi-automatic method.^{36,37}

Aortic calcification *in vitro*

Aortas were cultured from adult, male Sprague–Dawley rats as described earlier.⁷ Briefly, aortas were dissected from surrounding tissue and perfused with physiologic saline under sterile conditions. After the adventitia was carefully removed with scissors to avoid any mechanical trauma, 2–3 mm rings were placed in high-phosphate DMEM medium containing 3.75 U/ml of calf intestinal alkaline phosphatase and incubated at 37°C in 5% CO₂ with medium changes every 3 days. In studies comparing potencies of bisphosphonates, 25 µM warfarin was added to maximize calcification. Calcification was also produced by rubbing aortas with a cotton swab followed by culture without alkaline phosphatase. The total concentrations of calcium and phosphate were 1.8 and 3.8 mM respectively. To quantitate calcification, ⁴⁵Ca was added to the medium and incorporation into the aortic rings was measured after extracting the rings in 3 M HCl.

Calcium release

Injured aortas were cultured for 9 days in high-phosphate DMEM containing ⁴⁵Ca. The aortas were washed several times in low-phosphate DMEM and then placed in the same medium for 4 h to wash out unbound ⁴⁵Ca. Medium was then changed and samples were removed at the indicated times for measurement of ⁴⁵Ca. After 5 days, ⁴⁵Ca content of the aortas was measured as above.

Pyrophosphate hydrolysis

This was measured as described earlier⁹ by incubating freshly isolated aortas with 1 µM sodium pyrophosphate and [³²P]PPi (3 µCi/ml) and then adding ammonium molybdate (to bind orthophosphate) followed by extraction with isobutanol/petroleum ether (4:1) to separate phosphomolybdate from pyrophosphate. The rings were then dried and weighed.

Quantitative polymerase chain reaction

Aortas were frozen in liquid nitrogen, powdered and then extracted with a guanidinium and phenol solution on ice with additional homogenization. Chlorophorm was added and RNA extracted by centrifugation. cDNA was synthesized using 2 µg of total RNA from each sample with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and quantitative PCR performed with an iCycler iQ Detection System using iQ SYBR[™] Green Supermix (Bio-Rad). To avoid amplification of genomic DNA, primers for QPCR were designed to flank at least on intron. Sequences of the primer pairs (F: forward; R: reverse) were as follow. Osteopontin: CTGTCTCCCGGTGAAAGTG (F), GTCATCCGTTTCTTCAGAGG (R); matrix gla protein: AGGCAGACTCACAGGACACC (F), CATTTCTCCGGTTGGTGAAG (R); Runx 2: CTTACAAATCCTC CCAAG (F), GAGGCGGTACAGAAACAAAC (R); Osterix: TCTC CATCTGCCTGACTCCT (F), TTCTTTGTGCCTCCTTTTCC (R).

Statistics

The Mann–Whitney *U*-test was applied to nonparametric data (aortic calcium content and bone parameters). Differences between groups for all other data were examined by Student's *t*-test.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported by Grant DK069681 from the National Institutes of Health and a grant from the Genzyme Renal Innovations Program (to Dr O'Neill), and Nephrology Fellowship and Junior Faculty Grants from Amgen (to Dr Lomashvili). Portions of this work have previously been presented in abstract form at the American Society of Nephrology Annual Meeting 2006 and 2008.

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